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EXHIBIT RAS-2

This is exhibit RAS-2 referred to in Declaration Under 37 C.F.R. 1.132 by Richard Anthony Strugnell dated 27.9.07

Richard Strugnell

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Visualization of Aβ42(43) and Aβ40 in Senile Plaques with End-Specific Aβ Monoclonals: Evidence That an Initially Deposited Species Is Aβ42(43)

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Summary

To learn about the carboxy-terminal extent of amyloid β-protein (Aβ) composition of senile plaques (SPs) in the brain affected with Alzheimer's disease (AD), we employed two end-specific monoclonal antibodies as immunocytochemical probes: one is specific for Aβ40, the carboxyl terminus of Aβ1–40, while the other is specific for Aβ42(43). In the AD cortex, all SPs that were labeled with an authentic antibody were Aβ42(43) positive, while only one-third of which, on the average, were Aβ40 positive. There was a strong correlation between Aβ40 positivity and mature plaques. Two familial AD cortices with the mutation of β-amyloid protein precursor 717 (βAPP717) (Val to Ile) showed a remarkable predominance of Aβ42(43)-positive, Aβ40-negative plaques. Diffuse plaques, representing the earliest stage of Aβ deposition, were exclusively positive for Aβ42(43), but completely negative for Aβ40.

Introduction

Alzheimer's disease (AD) is pathologically characterized by innumerable senile plaques (SPs), neurofibrillary tangles, and loss of subsets of neurons throughout the cortex (reviewed by Selkoe, 1991). SPs are composed of amyloid β-protein (Aβ), a 39–43 amino acid peptide that is proteolytically derived from a large membrane-spanning glycoprotein referred to as β-amyloid protein precursor (βAPP; Kang et al., 1987).

With specific Aβ immunostaining, three types of SPs are distinguishable (Yamaguchi et al., 1988; Masliah et al., 1990): diffuse plaques, which stained as amorphous zones without distinct boundaries; immature plaques, which are well-defined usually spherical plaques containing swollen neurites; and mature (cored) plaques, which are well-circumscribed, compact, round amyloid cores with peripheral coronas containing swollen neurites. Among these three types, considered to represent the earliest stage of SP are diffuse plaques, at least a fraction of which may evolve into immature plaques and finally mature plaques.

Aβ deposition is thought to be primarily involved in the pathogenesis of AD because in Down's syndrome diffuse plaques precede other AD-specific neuropathological changes (Mann and Esiri, 1988; Mann, 1989) and, most importantly, because several kinds of βAPP mutations are found to cosegregate with overt clinical manifestations in certain early-onset familial AD (FAD) (Goate et al., 1991; reviewed by Mullan and Crawford, 1993a). Aβ deposited in AD brain has been reported to have some heterogeneities in its carboxyl terminus; Aβ1–42 appears to be the major species in the parenchymal deposition (Kang et al., 1987; Miller et al., 1993; Roher et al., 1993a), although one report claimed that Aβ1–40 is the major species (Mori et al., 1992). Regarding the vascular amyloid, there have been somewhat conflicting data (Joachim et al., 1988; Prelli et al., 1988; Miller et al., 1993), but a carefully conducted work has indicated that both species, Aβ1–40 and Aβ1–42, are equally abundant (Roher et al., 1993b). Although Aβ has been well known for its apparent insolubility (Masters et al., 1985), it has recently been shown that cultured cells constitutively secrete soluble Aβ (Haass et al., 1992; Shoji et al., 1992), the major species of which is Aβ1–40 (Seubert et al., 1992; Dovey et al., 1993). In particular, Aβ1–40 is a major form in the cerebrospinal fluid (Vigo-Pelfrey et al., 1993). Here, it should be noted that there is a difference by two or three amino acids between deposited and soluble forms of Aβ. This small difference would be significant: in vitro model experiments clearly showed that Aβ1–42 can polymerize into amyloid fibrils at a much faster rate than Aβ1–40, suggesting that the presence of these two carboxy-terminal amino acids, Ile and Ala, is critical for β-amyloidogenesis (Jarrett et al., 1993). This raises the possibility that an increased production or a decreased clearance of Aβ1–42, rather than Aβ1–40, is a determinant for amyloid formation.

For immunocytochemical probes, we developed two end-specific Aβ monoclonals: one is specific for the carboxyl terminus of Aβ1–40, Aβ40, while the other is specific for Aβ42(43). With these monoclonals, we have clearly shown the following: that Aβ42(43) is the major species in SPs in situ and is by far the most predominant in SPs of the brains with the βAPP717 (Val to Ile) mutation, and that diffuse plaques contain only Aβ1–42(43) species, but not Aβ1–40.

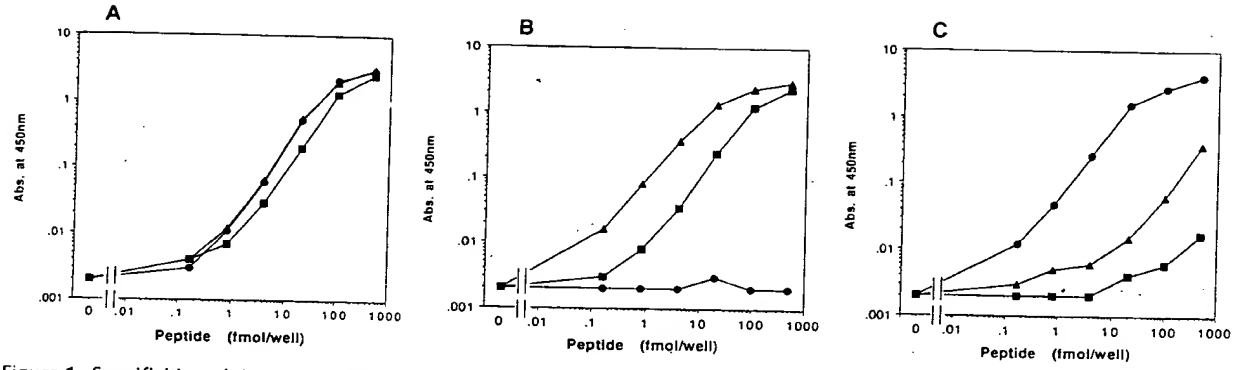

Figure 1. Specificities of the Three Aβ Monoclonals As Shown by EIA

Each amount of Aβ1–40 (closed circle), Aβ1–42 (closed triangle), or Aβ1–43 (closed square) was placed onto a BAN-50-coated microplate. Bound antigen was detected by horseradish peroxidase–labeled BS85 (A), BC05 (B), or BA27 (C) (see text for detailed procedures). Abbreviation: Abs, antibodies.

Results

Characterization of Three Monoclonal Antibodies to the Carboxyl Termini of Aβ

Specificities and sensitivities of the three monoclonals, BS85, BC05, and BA27, were examined by two-site enzyme immunoassays (EIAs) using several synthetic Aβ peptides with various carboxyl termini. Thus far, four different carboxyl termini, Aβ39 (Prelli et al., 1988), Aβ40 (Joachim et al., 1988; Mori et al., 1992; Miller et al., 1993; Roher et al., 1993b), Aβ42 (Kang et al., 1987; Miller et al., 1993; Roher et al., 1993a), and Aβ43 (Kang et al., 1987; Mori et al., 1992), have been reported in the parenchymal and vascular amyloid. BS85 showed almost the same sensitivities toward Aβ1–38, Aβ1–39 (data not shown), Aβ1–40, Aβ1–42, and Aβ1–43 (Figure 1A). Thus, this monoclonal can be used as an authentic probe for all Aβ species with different carboxyl termini. BC05 reacted preferentially with Aβ1–42, followed by Aβ1–43, but not with Aβ1–40 (Figure 1B). BA27 was 100- to 1000-fold more specific for Aβ1–40 than for other species, in particular, Aβ1–42 or Aβ1–43 (Figure 1C). The specificities of BC05 and BA27 were also assessed immunocytochemically by the absorption with Aβ peptides. BC05 immunostaining in tissue sections was eliminated by absorption with Aβ1–42 at a molar ratio of more than 500, while a molar ratio of 10× that of Aβ1–42 was required for complete absorption with Aβ1–43, which is consistent with the EIA assessment (Figure 1B). Aβ1–40, Aβ38–44, Aβ39–45, Aβ40–46, and Aβ41–47 were unable to affect the staining with BC05 at a molar ratio up to 10,000. Specific staining with BA27 was abolished with Aβ1–40 at a peptide-to-antibody molar ratio of more than 1000, but not with Aβ1–42 or Aβ1–43. These results further confirm that BC05 and BA27 are specific probes for Aβ42(43) and Aβ40 in the tissues, respectively.

BC05-Positive SPs Are Predominant over BA27-Positive SPs in Sporadic AD Cortex

Three consecutive tissue sections from temporal cortices of 10 sporadic AD cases were stained with BS85, BC05, and BA27. BS85-positive SPs were invariably positive for BC05, strongly suggesting that BC05 labels all of the SPs (Figures 2A and 2B). In fact, the staining with BS85 was found to be very similar to that with a polyclonal toward Aβ1–28 or Aβ1–40 (data not shown). On the morphological basis, SPs are classified into three categories: diffuse plaques, immature plaques, and mature (cored) plaques (Masliah et al., 1990). BC05 stained SPs rather uniformly; uncored plaques (diffuse and immature plaques) and mature ones were stained to similar intensities (Figure 2B). In mature

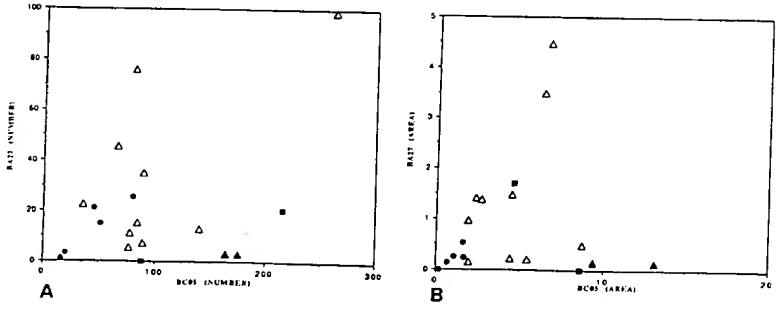

Figure 3. Morphometry of BC05- and BA27-Positive SPs

(A) The abscissa and ordinate represent the numbers of BC05-positive and BA27-positive SPs per square millimiter, respectively. (B) The abscissa and ordinate represent the percentages of the areas covered by BC05-positive and BA27-positive SPs, respectively. Closed circle, age-matched control; open triangle, sporadic AD; closed square, Down syndrome; closed triangle, FAD with βAPP717 mutation.

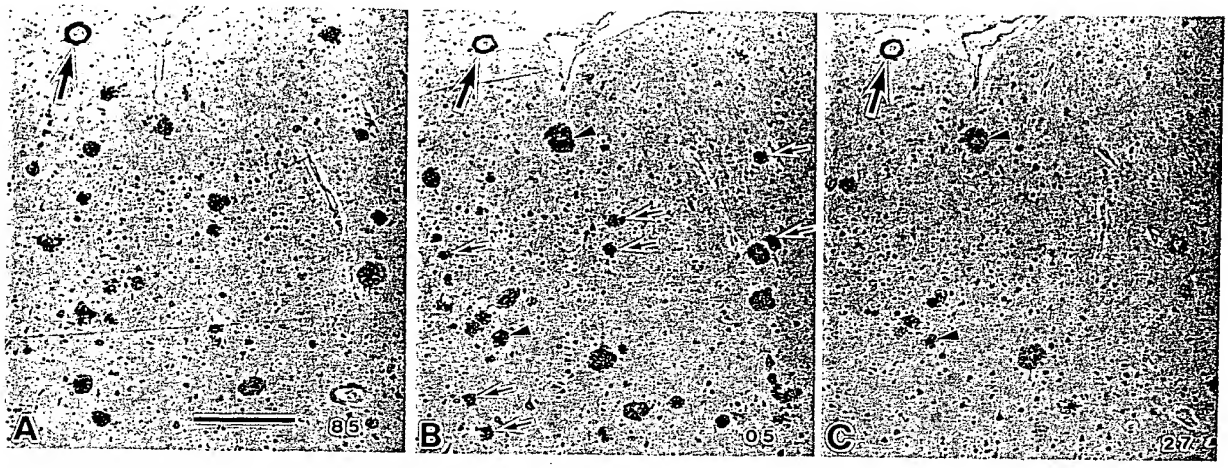

Figure 2. Immunostaining of SPs in the Inferior Temporal Cortex from a Sporadic AD Brain

Three consecutive sections were stained with BS85 (A), BC05 (B), and BA27 (C). Note that BS85 and BC05 label almost the same numbers of SPs, while BA27 labels only a subset of them; many BC05-positive SPs (arrows in [B]) are not stained with BA27 (C). BA27, but not BC05, stains the core portion (arrowheads in [C]) more intensely than the peripheral portion. An amyloid-bearing vessel (large arrows in [A, B, and C]) is positive for all three monoclonals. Scale bar equals 500 μm.

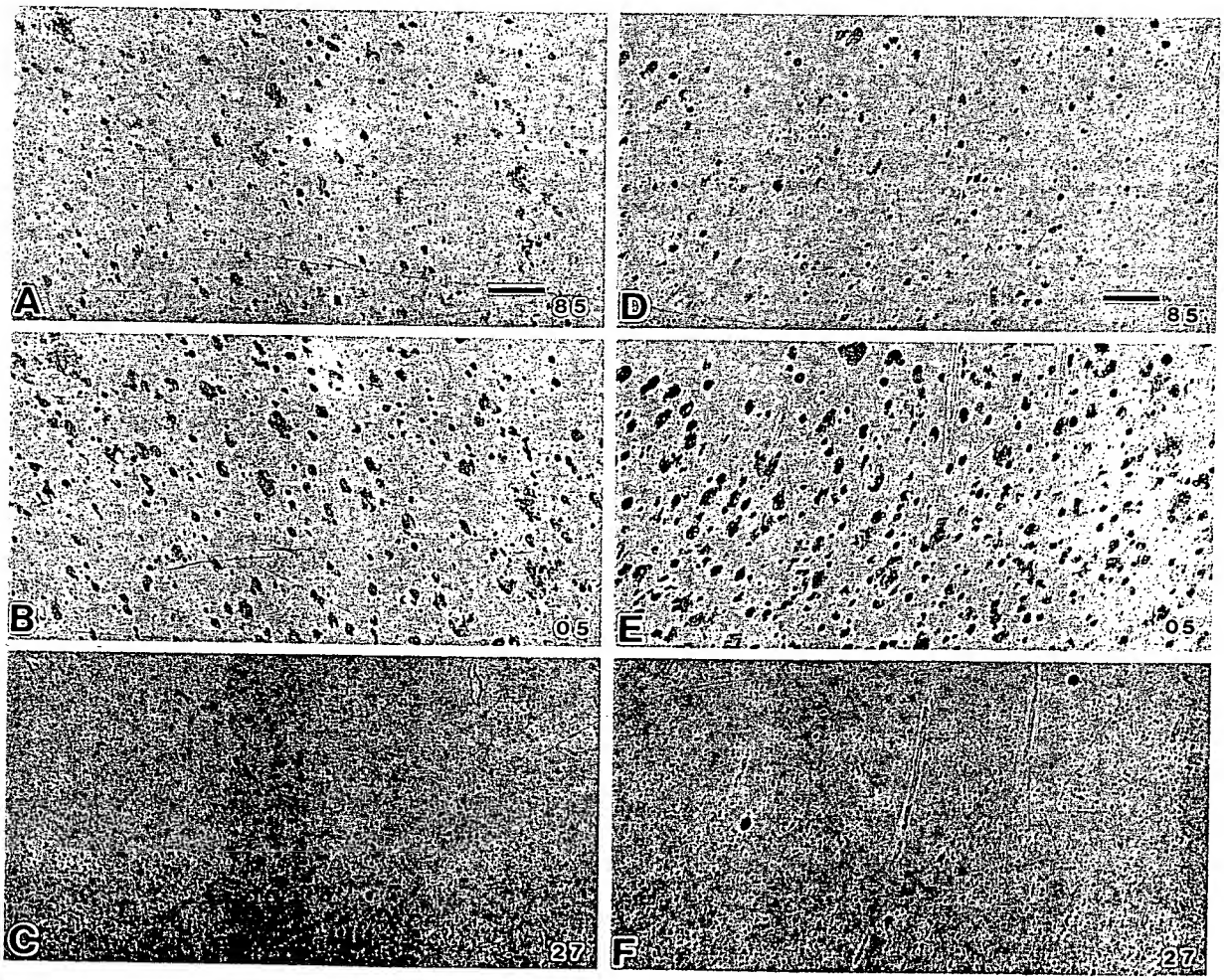

Figure 4. Immunostaining of SPs in FAD Brains with the βAPP717 (Val to Ile) Mutation

Innumerable SPs in the inferior temporal cortex are BC05 positive (B and E), but very few of them are BA27 positive (C and F). BC05 (B and E) and BS85 (A and C) label essentially the same numbers of SPs. (A–C) show case 1; (D–F) show case 2. Scale bar equals 200 μm.

(cored) plaques, core and halo portions were equally labeled. In contrast, BA27 labeled only a subset of (immature and mature) plaques and stained their portions unevenly; in the case of mature plaques, core portions were intensely labeled, but halo portions were either only weakly stained or not stained at all (Figure 2C). A substantial proportion of uncored plaques was only very weakly or barely stained (see below). Amyloid-bearing meningeal vessels were immunostained with all three monoclonals (Figures 2A–2C). SPs with amyloid-bearing vessels (drusige Entartung) were often strongly positive for BA27. SPs in nondemented aged cortices also showed the same staining characteristics (data not shown), although their number is quite small (Figures 3A and 3B). Without formic acid pretreatment, BA27 or BC05 labeled SPs only faintly, but the number of labeled SPs was essentially the same as that in the pretreated section (data not shown). This excludes the possibility that formic acid selectively removed Aβ1–40 from SPs.

A set of BC05- and BA27-stained sections from each inferior temporal cortex was subjected to morphometric analysis. The number of BC05-positive SPs ranged from 36–261/mm², with an average of 100, while that of BA27-positive SPs ranged from 5–98/mm² (mean of 33; Figure 3A). The area occupied by BC05-positive SPs amounted to 2.0%–8.7% (mean of 4.5%) of a given cortical area, while BA27-positive SPs covered 0.2%–4.5% (mean of 1.4%; Figure 3B). The ratio of BA27- to BC05-positive SPs ranged from 7.0%–93% (mean of 35%) on the number basis, while it ranged from 3.7%–68% (mean of 31%) on the area basis. Thus, roughly, only one-third of BC05-positive SPs were BA27 positive.

The relationship of BA27-positive plaques to plaque type was also investigated. In 10 AD cases, mature (cored) plaques were 0%–51% (mean of 23%) of SPs, and the rest were uncored (diffuse and immature) plaques. Among mature plaques, 0%–100% (mean of 62%) were BA27 positive. In contrast, among uncored plaques, 1.6%–62% (mean 33%) were BA27 positive.

Marked Predominance of BC05-Positive, BA27-Negative SPs in the Brain with βAPP717 (Val to Ile) Mutation

Two FAD cases from unrelated pedigrees, which were shown to carry the βAPP717 (Val to Ile) mutation, were similarly investigated; case 1, a 52-year-old female, presented a 4 year history of dementia (Naruse et al., 1991) and case 2, a 47-year-old male, had an 8 year history of dementia. Neuropathological examination of the two cases showed that AD pathologies were severe but qualitatively similar to those found in sporadic AD cases (Lantos et al., 1992; Mullan et al., 1993b).

Immunocytochemistry with BC05 and BA27 revealed remarkable predominance of BC05-positive, BA27-negative SPs in those two FAD cortices (Figures 4A–4F). SPs were far more abundant in those cases, and uncored (immature and diffuse) plaques were by far the most predominant species (Figures 4A–4F). Only a very small fraction of SPs was BA27 positive (Figures 4C and 4F). Histometric analysis indicated that the area and number of BC05-positive SPs were significantly greater than those in sporadic AD brain: 177/mm² and 9.4% in case 1 and 166/mm² and 13.2% in case 2, respectively (see Figures 3A and 3B). In contrast, those indices for BA27-positive SPs were found to be much less than those in sporadic AD brain: 3/mm² and 0.15% in case 1 and 3/mm² and 0.13% in case 2, respectively (see Figures 3A and 3B). As a result, the ratios of BA27- to BC05-positive SPs are extremely small: 1.7% in the number quotient and 1.6% in the area quotient in case 1 and 1.8% and 1.0% in case 2, respectively. Thus, the SPs in the βAPP717 mutation are characterized by both marked predominance of Aβ42(43) and scarcity of Aβ40.

Diffuse Plaques Are BC05 Positive but BA27 Negative

Although a great number of amorphous plaques resembling diffuse plaques were stained with BC05 in AD cortices, it was uncertain whether they are diffuse plaques as originally defined: that is, as faintly stained amorphous plaques without degenerating neurites (as seen by Bodian staining) (Yamaguchi et al., 1988). We therefore chose the following for immunostaining: a neocortex from a young Down's patient (Mann and Esiri, 1988) and cerebella (Joachim et al., 1989; Yamaguchi et al., 1989a) and corpora striata (Suenaga et al., 1990) from AD brains. The great majority of the SPs found in those areas are known as diffuse plaques. All of the diffuse plaques examined were BC05 positive, and the staining was found to be almost identical to that with BS85 (Figures 5A–5D; Figures 6A, 6B, 6D, and 6E). However, BA27 failed to stain BC05-positive diffuse plaques (see Figures 5E and 5F; Figures 6C and and 6F), whereas it stained vascular amyloid or mature plaques on the same sections (see Figure 5E; Figure 6C).

Discussion

Using end-specific Aβ monoclonals, we have immunocytochemically characterized SPs in situ as to whether SPs are composed of Aβ1–40 or Aβ1–42(43), whether SPs in the brain with the βAPP717 mutation are distinct from those of sporadic AD brain, and finally, whether the diffuse plaques, representing an initial stage of SPs, consist of Aβ1–40 or Aβ1–42(43). Although immunocytochemical studies are only qualitative in approach, they are quite important for the present purpose because of the following reasons: first, AD brain is very often complicated with cerebral amyloid angiopathy, which is invariably associated with such a large amount of Aβ1–40 (Joachim et al., 1988; Miller et al., 1993; Roher et al., 1993b; Suzuki et al., unpublished data) and Aβ1–42(43) (Roher et al., 1993b; Suzuki et al., unpublished data) that even a little contamination, which is inevitable, leads to incorrect values in the biochemical analysis. This means that the amount of Aβ1–40 or Aβ1–42(43) in a given AD brain tissue cannot be simply assumed to be derived

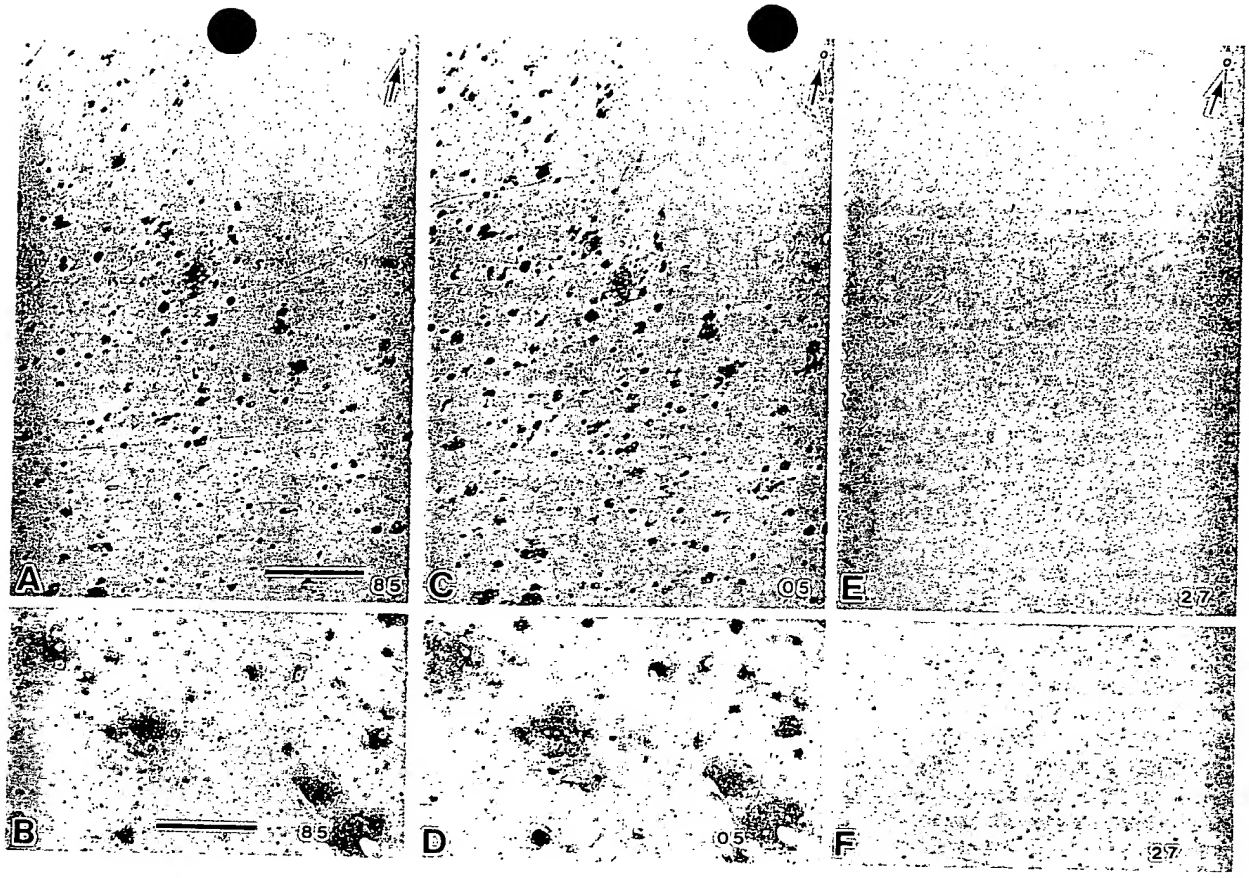

Figure 5. Immunostaining of Diffuse Plaques in a Down's Syndrome Brain

Diffuse plaques in the inferior temporal cortex are stained with BS85 (A and B) and BC05 (C and D) in an identical manner, but not with BA27 (E and F). Note that an amyloid-bearing vessel (arrows in [A, C, and E]) in the subarachnoidal space is positive for all three monoclonals. Scale bar equals 500 μm in (A, C, and E) and 200 μm in (B, D, and F).

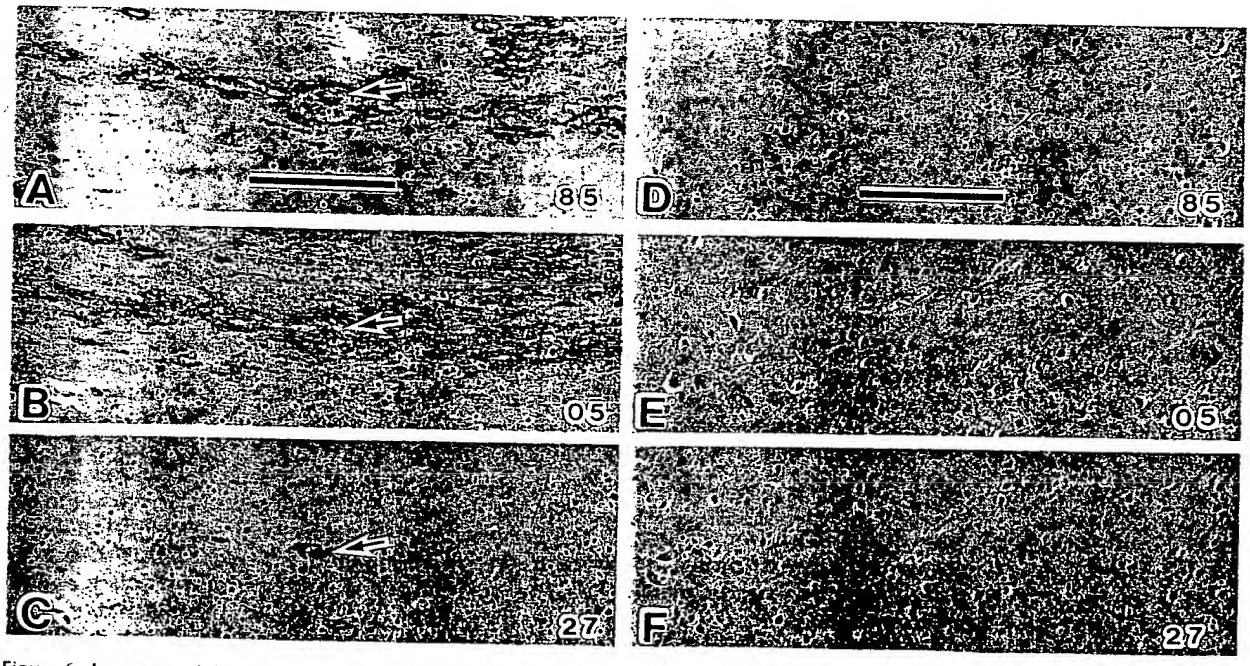

Figure 6. Immunostaining of Diffuse Plaques in Cerebellum and Striatum from a Sporadic AD

Diffuse plaques in the molecular layer of cerebellum (A to C) and putamen (D to F) are positive for BS85 (A and D) and BC05 (B and E), but negative for BA27 (C and F). However, a cored plaque (arrows in [A, B, and C]) in the cerebellum is labeled with BA27 (C). Scale bar equals 200 μm.

from parenchymal SPs. Thus, cerebral amyloid angiopathy would contribute to misleading results in the quantitation of Aβ1–40 and Aβ1–42(43) in the brain tissue and, thus, in the significance of each Aβ species; and second, diffuse plaques consist of scattered individual amyloid fibrils between unidentified cellular processes (Yamaguchi et al., 1989b; Davies and Mann, 1993) and appear to contain minute amounts of Aβ that escape detection by standard biochemical procedures (unpublished data). Thus, the present immunocytochemical approach provides us with the results that would not have been obtained from direct biochemical analyses.

The three monoclonals employed here have distinct specificities toward Aβ1–40, Aβ1–42, and Aβ1–43 (Figures 1A–1C). In EIA, BC05 reacted with both Aβ1–42 and Aβ1–43, but not with Aβ1–40. It is quite possible that BC05 preferentially labels Aβ42 rather than Aβ43 in SPs in situ (Figure 1B). However, to address this issue, we need another end-specific monoclonal differentiating Aβ42 from Aβ43. In addition, it should be noted that BC05 negativity does not exclude the possibility that an Aβ species longer than 44 residues is involved, although the presence of such longer Aβ species has not been definitely shown (Miller et al., 1993).

With the above results taken into account, the present immunocytochemical work has clearly shown that Aβ42(43)-positive SPs are the major species in sporadic AD brain, which is consistent with most previous and recent biochemical analyses (Kang et al., 1987; Miller et al., 1993; Roher et al., 1993a); in FAD brains with the βAPP717 mutation (Val to Ile), Aβ42(43)-positive, Aβ40-negative SPs were by far the most predominant, and Aβ40-positive SPs are very few; and diffuse plaques are Aβ42(43) positive but Aβ40 negative.

It is noteworthy that only a subset of SPs was Aβ40 positive, which was found much more frequently in mature (cored) plaques than in uncored (diffuse and immature) plaques. The presence of Aβ40-positive amyloid appears to be related to the evolution of SPs; at least a fraction of uncored plaques is considered to evolve into mature (cored) plaques during the course of AD, which may take more than a decade (Mann, 1989). Thus, it is quite likely that Aβ42 is processed to Aβ40 in situ, possibly by a carboxypeptidase, as a given plaque is getting older. Perhaps activated microglia, invariably accompanying mature (cored) plaques (Ohgami et al., 1991), may have a significant role for the removal of carboxy-terminal two or three amino acids. Related to this, it was previously reported that the antiserum to Aβ28–40 stained a fraction of SPs (Spillantini et al., 1990), which was attributed to the conformational changes of Aβ during plaque maturation. Alternative explanation would be that the antiserum was specific for the carboxyl end of Aβ28–40.

There is a further implication in this observation. It has recently been shown that externally added Aβ1–40 accumulates in a highly specific manner onto SPs and vascular amyloid in situ (Maggio et al., 1992), and this accumulation may significantly contribute to the growth of SPs in situ. This is indeed consistent with the "seeding" hypothesis (Jarrett et al., 1993); once a seed, presumably Aβ1–42, is formed, a major soluble species, Aβ1–40, is incorporated and SPs grow. However, if this is the case, all Aβ42(43)-positive plaques should contain Aβ40-positive amyloid fibrils because Aβ1–40 is the major species in the extracellular fluid or cerebrospinal fluid (Seubert et al., 1992; Dovey et al., 1993; Vigo-Pelfrey et al., 1993). The present observation that only a fraction of SPs is Aβ40 positive indicates that Aβ1–40 in the extracellular fluid does not have a significant role in the growth of SPs in situ.

Previous neuropathological examinations on FAD brains with the βAPP717 mutation demonstrated no distinct quantitative or qualitative difference from sporadic AD (Lantos et al., 1992; Mullan et al., 1993b). Furthermore, recent studies on neuroblastoma cell lines transfected with the mutated βAPP cDNA showed that the mutation does not increase the total amount of secreted Aβ (Cai et al., 1993). Thus, the situation is quite different from that of cells expressing doubly mutated βAPP that was found in a Swedish FAD family (Mullan et al., 1992): the mutation causes a striking 5- to 8-fold increase in Aβ secretion in vitro (Citron et al., 1992; Cai et al., 1993), which is currently assumed to be a cause of accelerated β-amyloidogenesis (Jarrett and Lansbury, 1993). From our immunocytochemical observations alone, we do not exactly know why the βAPP717 mutation shows such unusual predominance of Aβ42(43)-positive, Aβ40-negative SPs. One possibility would be that the mutation (at Aβ46) alters the preferred cleavage site of βAPP, which results in the increase of the Aβ1–42(43) species. In fact, it has very recently been shown that the proportion of Aβ1–42(43) secreted by neuroblastoma cells transfected with mutated βAPP717 (Val to Ile) cDNA is increased 1.5- to 1.9-fold (Suzuki et al., 1994).

We now look into the significance of the scarcity of Aβ40-positive SPs, which is considered to be the other characteristic of those brains. The carboxy-terminal processing of Aβ42(43) to generate Aβ40 could be so slow as compared with the rate of accumulation of Aβ1–42(43) that brains with the βAPP717 mutation have such unusual characteristics. The two FAD cortices contained abundant uncored types of SPs, most of which closely resemble diffuse plaques. If a large proportion of these uncored plaques represent diffuse ones, a marked predominance of Aβ42(43)-positive, Aβ40-negative SPs can be explained (see below). In this context, it is tempting to postulate that abundance of Aβ42(43)-positive, Aβ40-negative SPs reflects the amyloidogenesis activity in AD brain.

The present observation on diffuse plaques indicates that Aβ deposition begins most likely with Aβ1–42(43), and not with Aβ1–40, although we cannot exclude the possibility that a longer Aβ species is also involved. Thus, the central question in β-amyloidogenesis is as follows: how is Aβ1–42(43) produced and deposited? Aβ1–42(43), constitutively secreted (Cheung

et al., 1994) or abnormally cleaved off, may interact with extracellular matrix and deposit. The initiation of β-amyloidogenesis may depend on local concentrations of Aβ1–42(43), alterations of extracellular matrix, or both.

In sum, the present work has clearly shown that the initial Aβ deposition begins not with Aβ1–40 but with Aβ1–42(43) or longer Aβ. Aβ42(43)-positive, Aβ40-negative plaques may represent early-stage SPs, and during the course of AD, a varying proportion of them acquires Aβ40 positivity, presumably by processing.

Experimental Procedures

Cases

Brain tissues used for the present study were from 10 cases of sporadic AD (age 55–89, with an average age of 71 ± 11), two FAD cases from unrelated pedigrees with the mutation βAPP717 (Val to Ile) (Naruse et al., 1991; Mullan et al., 1993b; see text), two cases of Down's syndrome (case 1, 36-year-old male; and case 2, 65-year-old male), and five cases of nondemented aged individuals with SPs (age 75–95, with an average age of 83 ± 7.6). The diagnosis of AD was made based on clinical and pathological findings using criteria established by the National Institutes of Health Neuropathology Panel (Khachaturian, 1985).

Peptides

Three immunogenic moieties, Aβ1–40, Aβ25–35, and Aβ35–43, were synthesized with an automated peptide synthesizer (Model 430A, Applied Biosystems, Foster City, California). Aβ1–40 and Aβ1–42 used for EIA and absorption studies in immunocytochemistry were obtained from Bachem Feinchemikalien AG (Bundendorf, Switzerland). Aβ1–43 is a gift from Dr. K. Sato of the Life Science Institute of Mitsubishi Chemical (Takashima et al., 1993). Crude Aβ1–43 was purified on a high-pressure liquid chromatography column (TSK gel Octadecyl-2PW, Tosoh, Tokyo, Japan) under alkaline (pH 8.9) conditions. Aβ38–44, Aβ39–45, Aβ40–46, and Aβ41–47 were obtained from Fujiya (Kanagawa, Japan); each Aβ peptide was extended with Lys-Lys at its amino terminus.

Monoclonal Antibodies and Two-Site EIAs

BA27 and BS85 were prepared as described elsewhere (Suzuki et al., submitted). In brief, BALB/c mice were immunized with bovine thyroglobulin-conjugated Aβ1–40 or Aβ25–35. BA27 and BS85 were selected from those monoclonals directed against Aβ1–40 and Aβ25–35, respectively. BC05 was similarly raised against thyroglobulin-conjugated Aβ35–43. Three monoclonals were purified from ascites with a protein A–immobilized column (IPA-300, Repligen, Cambridge, Massachusetts). The two-site EIA for Aβ was carried out in the same way as previously described (Suzuki et al., submitted), using BAN-50 (monoclonal to Aβ1–16) as an immobilized capture antibody and using horseradish peroxidase–labeled BS85, BA27, and BC05 as detector antibodies. Varying amounts of Aβ1–40, Aβ1–42, and Aβ1–43 were put in each well of a BAN-50-coated microtest plate (Immuno Plate I, Nunc, Roskilde, Denmark) and incubated at 4°C for 24 hr. After rinsing with phosphate-buffered saline, loaded wells were reacted at 4°C for 24 hr with appropriately diluted horseradish peroxidase–labeled BS85, BA27, or BC05. Bound-enzyme activity was measured by the TMB Microwell Peroxidase Substrate System (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland).

Immunocytochemistry

Brain tissues obtained at autopsy were fixed in 10% buffered formalin for 1–2 weeks. Specimens briefly (24 hr) fixed with 4% formaldehyde showed similar staining with three monoclonals. Blocks from various portions of brain were excised, dehydrated, and embedded in paraffin, and serial sections were cut at 6 μm thickness. Sections from the temporal lobe including hippocampal formation and inferior temporal gyrus were studied in all cases. Cerebella and corpora striata were examined in four AD cases. Sections were pretreated with 99% formic acid for 5 min before immunostaining (Kitamoto et al., 1987). Three consecutive sections were immunostained with BS85, BC05, and BA27 according to the avidin–biotin method using 3,3'-diaminobenzidine as chromogen and were lightly counterstained with hematoxylin. Counterstaining was omitted in sections for image analysis. BC05 was used at a concentration of 0.15 μg/ml, BA27 was used at 0.22–2.2 μg/ml, and BS85 was used at 2.8 μg/ml. For the absorption of immunostaining, BC05 or BA27 was incubated with Aβ1–40, Aβ1–42, and Aβ1–43, and BC05 was incubated with Aβ38–44, Aβ39–45, Aβ40–46, and Aβ41–47 at a peptide:antibody ratio (molar:molar) of 1:100 to 1:10,000. Each Aβ peptide (1 mg/ml) dissolved in dimethyl sulfoxide was mixed with diluted antibodies and incubated for 8 hr at room temperature and was then applied for immunocytochemistry.

Morphometric Analysis of SPs

The quantitation of SPs was carried out using an Olympus Image Analysis System (SP1000, Model 1500 C2 Olympus). Images were captured from a cortical area of 1.67 mm × 1.67 mm (2.8 mm^2) in the inferior temporal gyrus, which almost covers the entire depth of the cortex. SPs were identified by grey-scale thresholding, and artifacts were deleted by manual editing. The total number of SPs and the sum of the SP-covered area divided by the total area (see Hyman et al., 1993) were calculated in two consecutive, BC05- and BA27-stained sections. The ratios of BA27-positive to BC05-positive SPs were calculated on the number or area basis.

In each AD case, BC05- and BA27-positive plaques in the same field (approximately 2 mm^2) from adjacent sections were manually counted and classified into two types of SPs: uncored (diffuse and immature) plaques and mature (cored) plaques.

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